EXHIBIT A

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Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus Toxin and Botulinum Neurotoxin Type A*

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To define conserved domains within the light (L) chains of clostridial neurotoxins, we determined the sequence of botulinum neurotoxin type B (BoNT/B) and aligned it with those of tetanus toxin (TeTx) and BoNT/A, BoNT/C1, BoNT/D, and BoNT/E. The L chains of BoNT/B and TeTx share 51.6% identical amino acid residues whereas the degree of identity to other clostridial neurotoxins does not exceed 36.5%. Each of the L chains contains a conserved motif, HExxHxxH, characteristic for metalloproteases. We then generated specific 5′- and 3′-deletion mutants of the L chain genes of TeTx and BoNT/A and tested the biological properties of the gene products by microinjection of the corresponding mRNAs into identified presynaptic cholinergic neurons of the buccal ganglia of *Aplysia californica*. Toxicity was determined by measurement of neurotransmitter release, as detected by depression of postsynaptic responses to presynaptic stimuli (Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., and Tauc, L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 7844–7848). Our studies allow the following conclusions. 1) Residues Cys^{435} of TeTx and Cys^{430} of BoNT/A, both of which participate in the interchain disulfide bond, play no role in the toxification reaction. 2) Derivatives of TeTx that lacked either 8 amino- or 65 carboxyl-terminal residues are still toxic, whereas those lacking 10 amino- or 68 carboxyl-terminal residues are nontoxic. 3) For BoNT/A, toxicity could be demonstrated only in the presence of added nontoxic heavy (H) chain. A deletion of 8 amino-terminal or 32 carboxyl-terminal residues from the L chain had no effect on toxicity, whereas a removal of 10 amino-terminal or 57 carboxyl-terminal amino acids abolished toxicity. 4) The synergistic effect mediated by the H chain is linked to the carboxyl-terminal portion of the H chain, as demonstrated by injection of H_C-specific mRNA into neurons containing the L chain. This finding suggests that the H_C domain of the H chain becomes exposed to the cytosol during or after the putative translocation step of the L chain.

Tetanus toxin (TeTx)[1] and the seven serologically distinct botulinal neurotoxins, designated BoNT/A to BoNT/G, are synthesized as single-chain polypeptides (M_r 150,000). Proteolytic activation generates di-chain species in which the L chains (M_r = 50,000) are disulfide linked to the corresponding H chains (M_r 100,000) which provide the machinery for neuroselective binding, internalization, and translocation of the toxic L chains into the cytosol.

Although TeTx resembles BoNTs in regard to biosynthesis and molecular architecture (Fig. 1), the primary site of action differs for the two classes of neurotoxins, and therefore different clinical manifestations arise: the spastic disorder caused by TeTx is caused by a blockade of central synapses, but the flaccid paralysis observed in botulism involves inhibition of neuromuscular junctions. The toxic action has been proposed to develop in three consecutive steps (Simpson, 1980; Schmitt *et al.*, 1981): binding of the toxins to neuronal membrane acceptors, internalization and intraneuronal targeting, and blockade of neurotransmitter release. The H chains bind to cellular receptors (Simpson, 1986) and mediate translocation of the corresponding L chains into the cytosol (Morris and Saelinger, 1989; Poulain *et al.*, 1991). Although the molecular mechanism(s) underlying toxification through the L chains remain obscure, it has been demonstrated convincingly that the L chains of TeTx and BoNT/A alone are sufficient to abolish catecholamine release from permeabilized bovine adrenal chromaffin cells used as a model for neuronal secretion (Ahnert-Hilger *et al.*, 1989; Bittner *et al.*, 1989). Central synapses in *Aplysia* have been found to be highly sensitive to clostridial neurotoxins (Poulain *et al.*, 1988; 1989b; Mochida *et al.*, 1989, 1990). As in vertebrate cells, a blockade of transmitter release was observed in the cholinergic synapses of buccal or in the non-cholinergic synapses of cerebral ganglia when the isolated L chain of TeTx was injected into presynaptic neurons (Mochida *et al.*, 1989; Poulain *et al.*, 1990). However, in this system toxicity of the L chains of BoNT/A or BoNT/B could be demonstrated only

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[1] The abbreviations used are: TeTx, tetanus toxin; BoNT/A–G, botulinum neurotoxin types A–G, respectively; L chain, light chain; H chain, heavy chain; H_C(BoNT/A), carboxyl-terminal fragment of the H chain comprising residues Asn^{872} to Leu^{1296} of BoNT/A; ACh, acetylcholine; PCR, polymerase chain reaction.

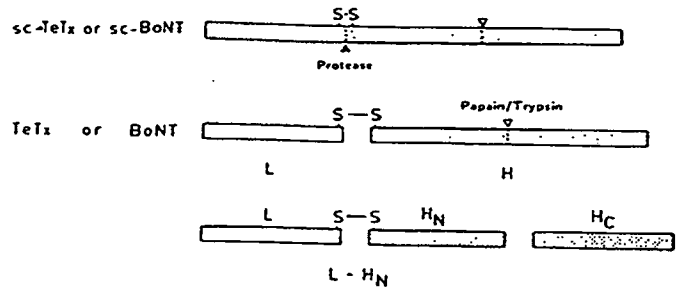


FIG. 1. Common structure, biosynthesis, and nomenclature for clostridial neurotoxins and their subfragments. Single-chain toxins (*sc-TeTx* or *sc-BoNT*), as synthesized by *Clostridium tetani* or *C. botulinum*, are activated by bacterial or host proteases into their di-chain neurotoxic isoforms. Proteolytic digestion with specific proteases *in vitro* may generate L H_N and H_C. Nomenclature according to Aguilera *et al.* (1992).

in the presence of the H chains which by themselves, however, are completely nontoxic (Poulain *et al.*, 1988).

We have previously cloned and sequenced the structural genes of TeTx (Eisel *et al.*, 1986), BoNT/A (Binz *et al.*, 1990a), BoNT/C1 (Hauser *et al.*, 1990), BoNT/D (Binz *et al.*, 1990b), and BoNT/E (Poulet *et al.*, 1992). In this study we present the sequence of the L chain of BoNT/B. An alignment of the individual L chain sequences reveals at least three highly conserved domains, suggesting that they should serve a conserved structural or biological function.

Each of the L chains contains, close to its COOH-terminal end, a single cysteine residue which mediates binding to the corresponding H chain. In this respect the clostridial neurotoxins resemble several postsynaptic snake venom neurotoxins all of which contain cysteine residues in this position (Endo and Tamiya, 1987). The L chain acts on permeabilized chromaffin cells only upon reductive separation from the H chain (Ahnert-Hilger *et al.*, 1989; Stecher *et al.*, 1989a), and reconstitution experiments between the two subchains indicated that the sulfhydryl group of this particular cysteine was indeed highly reactive (Weller *et al.*, 1988). Furthermore, Schiavo *et al.* (1990) have demonstrated that the interchain disulfide bond of TeTx is required for nerve cell penetration and that permeabilized cells are capable of reducing this bond. Therefore, it could be speculated that the newly generated free sulhydryl group within the carboxyl-terminal domain of the L chain could play a direct role in the toxification process.

In this study we have applied amino- and carboxyl-terminal deletion mutants of the L chains of TeTx and BoNT/A to define the minimal essential domains required for toxicity. Furthermore, we demonstrate that the unexplained synergistic role of the botulinal H chain is mediated by its H_C portion.

MATERIALS AND METHODS

Cloning and Sequencing of Gene Fragments Encoding the L chains of BoNT/E and BoNT/B—Cloning and sequencing of the entire BoNT/E gene from *Clostridium botulinum* (strain Beluga) (DDBJ, EMBL, and GenBank nucleotide sequence data base accession number X62089) and from two strains of *Clostridium butyricum* (strains ATCC 43181 and 43755) (accession number X62088) have been published recently (Poulet *et al.*, 1992). Cloning of chromosomal fragments from *C. botulinum* type B (strain Okra) encoding BoNT/B will be published elsewhere. To establish the sequence of the L chain, a 600-base pair *Dra*I fragment was identified with a synthetic 23-mer oligonucleotide, 5′-AATAATTTTAATTATAATGATCC-3′, the sequence of which corresponded to a sequence published previously for the amino-terminal region of the L chain of BoNT/B (Schmidt *et al.*, 1984). Hybridization conditions were as described (Binz *et al.*, 1990a). Subsequent clones were obtained by PCR and characterized by direct sequencing of both strands using the chain termination method (Sanger *et al.*, 1977).

Transcription Vectors Encoding L Chain Variants of TeTx and BoNT/A—To introduce a translational termination codon into the position exactly corresponding to the proteolytic cleavage site between the L and the H chains of TeTx, we applied site-directed mutagenesis (Taylor *et al.*, 1985). A 1.9-kilobase *Eco*RI/*Hin*dIII fragment from pEJ97 (Eisel *et al.*, 1986) was subcloned into M13mp19 (Yanisch-Perron *et al.*, 1985) and the codons GCA (for Ala^{457}) and TCA (for Ser^{458}) were changed into GCC (no amino acid exchange) or GGC (to yield Gly^{458}), respectively. The newly generated singular *Nae*I site was then used to insert the synthetic oligonucleotide 5′-TAGGATCCATG-3′ providing an in-frame termination codon for translation, a singular *Bam*HI site and an ATG codon for initiation of translation of the H chain. The orientation of the oligonucleotide was verified by sequencing. To generate an optimal Kozak's consensus sequence (Kozak, 1987) the *Sna*BI/*Bam*HI-fragment was treated with Klenow polymerase and cloned into the filled-in *Acc*I site of the pSP64 vector (Promega, Madison, WI) to yield pEN14. Through this operation a G residue was brought into the −3 position upstream from the ATG codon, as desired for optimal initiation of translation, and the *Bam*HI site was regenerated. The *Hin*dIII/*Bam*HI fragment from pEN14 was isolated, ends were filled in with Klenow polymerase, and the fragment was then cloned into the *Eco*RV site of a modified pSP73 vector (Promega; Krieg and Melton, 1988), to yield pSA14 (Fig. 2*A*). This vector contained an additional terminator sequence providing termination codons in all three reading frames, and a poly(dA-dT) segment derived from an influenza A virus hemagglutinin gene (Min Jou *et al.*, 1980) to allow for the synthesis of polyadenylated mRNA during *in vitro* transcription.

A similar vector, pBN1, was constructed for the synthesis of BoNT/A L chain-specific mRNA. A chromosomal 1,480-base pair *Ssp*I fragment (Binz *et al.*, 1990a) was cloned into the *Eco*RV site of pSP73 (Fig. 2*B*).

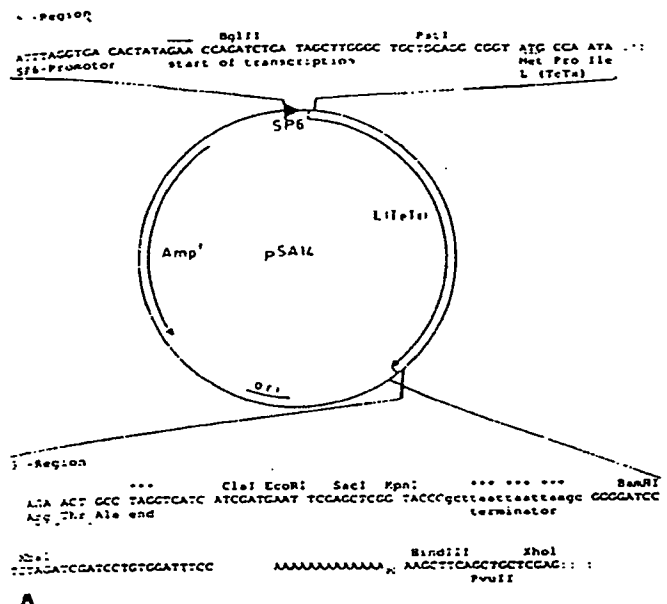
Construction of Deletion Mutants—Synthetic oligonucleotides and PCR methodology were applied to generate specific 5′deletions in the coding regions for the L chains of TeTx and BoNT/A. For TeTx upstream primers were: 5′-GAGAGAAGCTTGCCATGAGATATAGTGATCCTGTT-3′, 5′-GAGAGAAGCTTGCCATGAGTGATCCTGTTAATAAT-3′, 5′-GAGAGAAGCTTGCCATGCCTGTTAATAATGATACA-3′, 5′-GAGAGAAGCTTGCCATGATTATGATGGAGCCACCA-3′, and 5′-GAGAGAAGCTTGCCATGGTGCCGGAAAGGTATGAA-3′. The oligonucleotide 5′-CAGTTTTACCATGGTTTGTAAAAATC-3′, covering the *Nco*I site in position 550 of the published TeTx sequence (Eisel *et al.*, 1986) was used as a downstream primer. The PCR products were digested with *Hin*dIII and *Nco*I and ligated with the large fragment of pSA14 obtained by cleavage with the same enzymes.

For BoNT/A mutants the following synthetic oligonucleotides were used as upstream primers: 5′-GGGAGAGATCTGCCATGAATTATAAAGATCCTGTAAATGG-3′, 5′-GGGAGATCTGCCATGAAAGATCCTGTAAATGGT-3′, 5′-GGGAGATCTGCCATGCCTGTAAATGGTGTTGAT-3′, 5′-GGGAGAGATCTGCCATGGCTTATATAAAAATTCCA-3′, and 5′-GGGAGATCTGCCATGATTCCAGAAAGAGATACATT-3′. In this instance the oligonucleotide 5′-CTGTATAATATCAGCTGAGGGTCC-3′, containing the unique *Puv*II site in position 823 of the published BoNT/A sequence (Binz *et al.*, 1990a) was used as the second primer. All PCR products were reanalyzed by DNA sequencing.

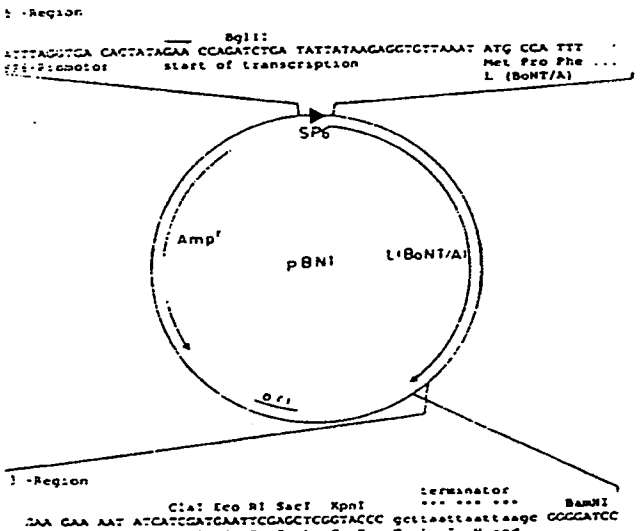
To generate 3′-deletions, pSA14- or pBN1-DNA (Fig. 2, *A* and *B*) was digested with *Eco*RI and *Kpn*I, treated for various lengths of time with exonuclease III, subsequently with S1 nuclease and Klenow polymerase (all enzymes from Promega, Heidelberg, FRG), and religated. Deletion mutants were characterized by direct sequencing using the synthetic oligonucleotide 5′-CAGGATCGACTCTAG-3′. Translations of the corresponding mRNAs were performed in rabbit reticulocyte lysate (Amersham, Braunschweig, FRG). The mutant peptides derived from individual clones are characterized in greater detail in Table II.

Recombinant H_C Fragment from BoNT/A—The synthetic oligonucleotides 5′-GAGAGAGGATCCATGAATATTATTAATACTTCTATATTG-3′ and 5′-GAGAGAGGATCCTTACAGTGGCCTTTCTCCCCATCC-3′ were used to amplify a gene fragment encoding the entire H_C region from BoNT/A. Chromosomal DNA (4 μg) from *C. botulinum* (strain 62A) served as a template in the protocol detailed below. The PCR product was then treated with *Bam*HI and cloned into *Bam*HI-digested pSP73 to yield pMQ9. The construct was resequenced and tested by combined *in vitro* transcription/translation.

Polymerase Chain Reactions—PCR reactions were performed in a



A



B

FIG. 2. Transcription vectors used for *in vitro* synthesis of mRNA encoding the L chains of TeTx (*A*) or BoNT/A (*B*) or various deletion mutants. Sequences of the 5'- and 3'-noncoding regions are shown in greater detail. Polyadenylation was guaranteed by the presence of a poly(dA-dT) segment located between the *Xba*I and the *Hin*dIII sites. This fragment was isolated from a cDNA clone encoding the hemagglutinin of an influenza virus (Min Jou *et al.*, 1980).

total volume of 50 μl containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.1% bovine serum albumin, a 100 μM concentration of each dNTP, 10 mM β-mercaptoethanol, 25 pmol of each primer, 2.5 units of Taq-polymerase (Boehringer, Mannheim, FRG), and 10 ng of pSA14 or pBN1 DNA previously linearized with *Xho*I. The reaction mixtures were denatured for 2 min at 93 °C and then subjected to 25 consecutive cycles consisting of denaturation (1 min at 93 °C), annealing (2 min at 55 °C), and polymerization (3 min at 73 °C). Amplified products were digested with the corresponding restriction endonucleases, purified by chromatography on QIAGEN columns (Diagen Düsseldorf, FRG), and characterized by DNA sequencing.

Preparation of L Chain-specific mRNAs—Protocols for *in vitro* synthesis of 5'-capped and 3'-polyadenylated RNA from recombinant pSP73 vectors have been published previously (Mayer *et al.*, 1988). For the synthesis of radiolabeled TeTx LC-specific mRNA, pSA14 was linearized with *Xho*I or *Bam*HI and transcribed with SP6 RNA polymerase (Bethesda Research Laboratories). For stability analyses of individual mRNA species, transcriptions were performed in the presence of 50 μCi of [α-^{32}P]ATP (3,000 Ci/mmol; Amersham).

Electrophysiological Analyses of Alterations Induced by Injection of L Chain-specific mRNA into Aplysia californica Presynaptic Neurons—Experiments were performed with buccal ganglia dissected from *A. californica* (Marinus Inc., Long Beach, CA) as described previously (Mochida *et al.*, 1990). Briefly, preparations were pinned in a 1-ml experimental chamber and superfused continuously (10 ml/h and 3 ml/min during washing steps) with artificial seawater (460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 25 mM $MgCl_2$, 28 mM $MgSO_4$, 10 mMTris/HCl, pH 7.8), except when the H chain of BoNT/A was added to the bath. Recordings were made from the couples of cholinergic neurons of the buccal ganglia which make well defined Cl^--dependent synapses (Tauc *et al.*, 1974). Both pre- and postsynaptic neurons were impaled with two microelectrodes (3 M KCl, 1.5–4 megohm). The presynaptic cells were current clamped to −50 mV and stimulated once/min. In this experimental setup, the amplitude of postsynaptic responses, as measured as current by conventional voltage-clamp technique and expressed as membrane conductances, is proportional to the amount of ACh released per impulse. mRNA (0.5 μg/μl) was injected into the presynaptic neuron by air pressure using an additional micropipette under visual and electrophysiological monitoring. To estimate the injected volume, the mRNA was mixed with a dye solution (1% (w/v) Fast Green FCF; Sigma). The injected sample volume (<0.4 nl) was between 2 and 10% of the cell body volume leading to a maximal theoretical intracellular concentration of L chain mRNA of 100 nM. After injection, the injection micropipette was removed.

RESULTS AND DISCUSSION

Alignment of the L Chain Amino Acid Sequences of Various Clostridial Neurotoxins—We compared by visual inspection the newly established amino acid sequence of the L chain of BoNT/B with those of BoNT/D (Binz *et al.*, 1990a), BoNT/C1 (Hauser *et al.*, 1990), BoNT/A (Binz *et al.*, 1990b), TeTx (Eisel *et al.*, 1986), and with the recently determined sequences of BoNT/E, as derived from *C. botulinum* and *C. butyricum* (Poulet *et al.*, 1992). The alignment in Fig. 3 shows at least four interesting features.

1. Three domains of increased homology can be coarsely defined based on the distribution of identical or similar amino acid residues. These domains comprise the 120 amino-terminally located residues, a central core region involving residues 186–241 of BoNT/B containing a highly conserved histidine-rich motif, HExxHxxH, characteristic for zinc-dependent metalloproteases, and a carboxyl-terminal domain consisting in the case of BoNT/B of residues 296–413. In metalloproteases, the first and the second histidine residue constitute zinc binding sites, whereas the glutamic acid residue provides the nucleophilic activity in the active center of the protease (Jongeneel *et al.*, 1989). From studies designed to map the epitopes of monoclonal anitbodies, we know that the conserved domains I and III fold by themselves into compact tertiary structures largely independent of the other two domains.[2] It remains to be shown whether carbonyl groups of residues Asp^{75}, Asp^{77}, Asn^{80}, Asp^{83}, or Glu^{266}, Glu^{267}, Asp^{232}, Glu^{234}, Asp^{240} of BoNT/B contribute to binding of Ca^{2+} ions, as observed in several thermolysin-related proteases (Stoeva *et al.*, 1990). Furthermore, although it has been demonstrated

[2] H. Kurazono, S. Kozaki, and H. Niemann, unpublished data.

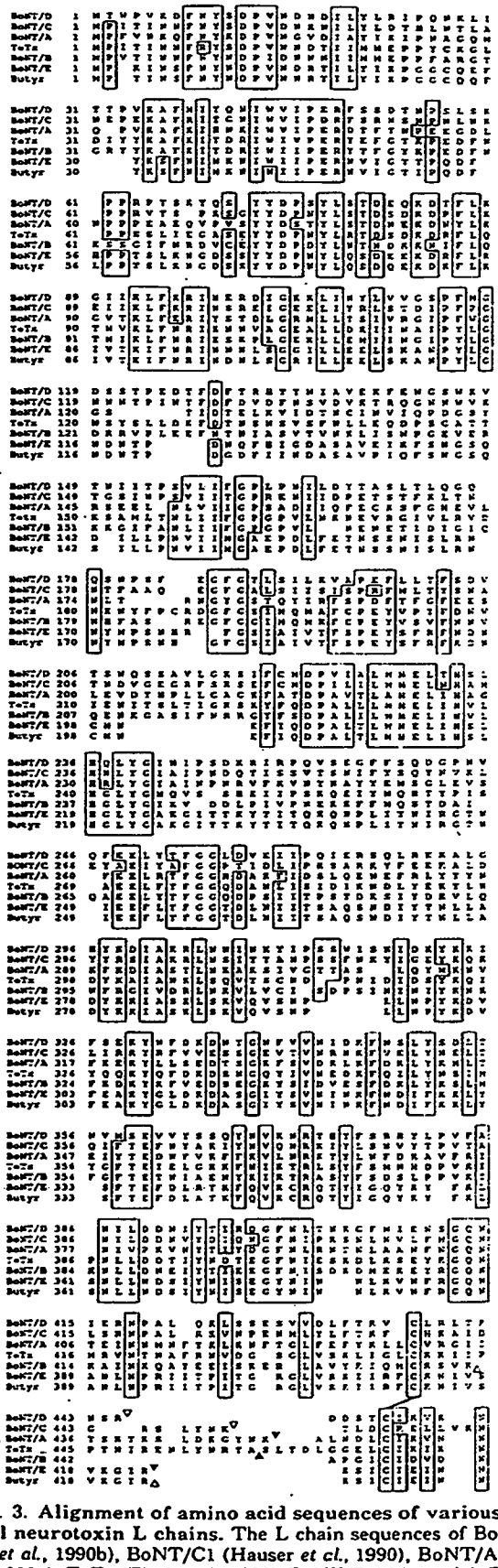


FIG. 3. **Alignment of amino acid sequences of various clostridial neurotoxin L chains.** The L chain sequences of BoNT/D (Binz *et al.*, 1990b), BoNT/C1 (Hauser *et al.*, 1990), BoNT/A (Binz *et al.*, 1990a), TeTx (Eisel *et al.*, 1986), BoNT/B (this study), BoNT/E from *C. botulinum* (strain Beluga) or *C. butyricum* (Butyr, ATCC strains 43755 and 43181, Poulet *et al.*, 1992) were aligned by visual inspection. Identical or related residues are *boxed*. Cysteine residues involved in the disulfide linkage between the L and the H chains are *interlinked*. *Filled arrowheads* specify proteolytic cleavage sites that have been verified previously by sequencing at the peptide level. *Open arrowheads* mark putative cleavage sites.

convincingly that various chelating agents detoxify clostridial neurotoxins (Bhattacharyya and Sugiyama, 1989), the cation(s) bound to the putative sites in the L chains have not yet been characterized unambiguously.

2. The L chain of BoNT/B is most closely related to that of TeTx (51.6% identity, Table I). This is in keeping with previous reports documenting that in the mouse motor nerve terminal TeTx and BoNT/B display electrophysiologically very similar inhibitory effects on the spontaneous and nerve-evoked transmitter release. Furthermore, the two toxins show similar sensitivity to 4-aminopyridine (Gansel *et al.*, 1987). Based on these and similar data, it has been suggested that TeTx or BoNT/B on the one side and BoNT/A on the other act on different intracellular targets (Gansel *et al.*, 1987; Dreyer *et al.*, 1987; Simpson *et al.*, 1988) This is further supported by the finding that the L chains of both TeTx and BoNT/B exhibit only about 32% identity in comparison with BoNT/A (Table I). It should be noted, however, that the high degree of relatedness between TeTx and BoNT/B only applies to the L chains and does not extend into the H chains where the degree of identity amounts to only 37.4% (data not shown).

3. Only 7 identical residues are found within the 55 carboxyl-terminal amino acids of the individual L chains. This low degree of identity continues within the amino-terminal portion of the individual H chains (not shown). It is, therefore, tempting to speculate that the nonessential carboxyl-terminal portion of the L chains merely serves a spacer function to provide flexibility for the postulated translocation of the L chains through an H chain-specified pore (Boquet and Duflot, 1982; Boquet *et al.*, 1984; Shone *et al.*, 1987; Hoch *et al.*, 1985; Donovan and Middlebrook, 1986).

4. The cysteine residues involved in the disulfide linkage between the L and the H chains of the individual toxins can be aligned. This results in nicking regions of various length comprising 8 amino acid residues in BoNT/B and 27 residues in TeTx. Analyses of the unnicked single-chain toxins for surface probability suggested, however, that in all instances the hinge regions are exposed at the surface of the toxin molecules. This would explain why this region is accessible for a variety of proteases (Weller *et al.*, 1988; Dekleva and DasGupta, 1990).

Stability of Microinjected mRNA—Previous studies (Mochida *et al.*, 1990) have shown that the molecular genetic approach involving microinjection of *in vitro* generated mRNA into identified presynaptic neurons of *A. californica* could provide a safe experimental access to gain further insights into the mechanism(s) by which the neurotoxins block neurotransmitter release. We were initially concerned about the stability of microinjected mRNA derived from clostridial genes containing about 73% A+T in the coding and about 80% in the noncoding regions (Binz *et al.*, 1990a, 1990b). Several studies on rapidly degraded mRNA have indicated that such degradation appears to be controlled by the presence of conserved A+U-rich sequences within the 3′-noncoding region (Brewer and Ross, 1988; Shaw and Kamen, 1986; Wilson and Treisman, 1988). The mRNA encoding the L chain of TeTx, for instance, contained within its 1,371 nucleotides eight copies of the motif 5′-AUUUA-3′ which has been proposed to constitute recognition sequences for an mRNA processing pathway that specifically degrades the mRNAs of

TABLE I

Degree of sequence identity and similarity between individual types of clostridial neurotoxins

% Similarity	% Identity					
	TeTx	BoNT/A	BoNT/B	BoNT/C1	BoNT/D	BoNT/E
TeTx	100	52.82	69.64	55.51	54.88	61.93
BoNT/A	32.05	100	53.38	52.64	54.38	53.24
BoNT/B	51.60	32.40	100	55.63	54.02	58.64
BoNT/C1	34.83	34.48	33.56	100	60.90	53.38
BoNT/D	34.47	34.10	34.48	46.52	100	55.01
BoNT/E	43.86	33.33	36.50	34.06	36.43	100

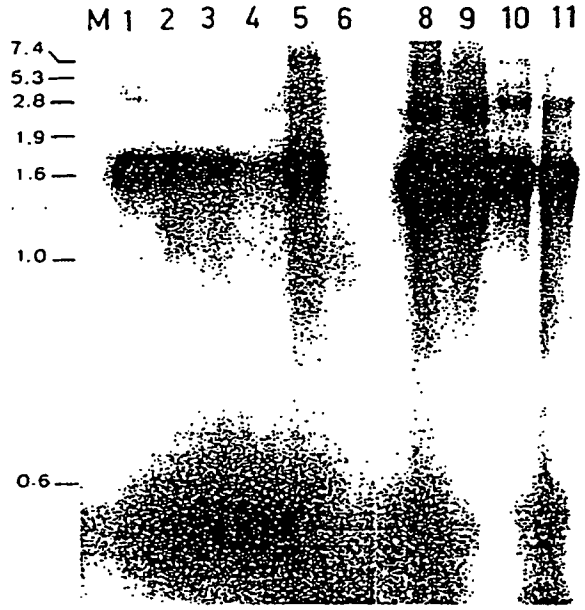


FIG. 4. Stability of microinjected TeTx L chain-specific mRNA in *Xenopus* oocytes. pSA14 was linearized with *Xho*I (*lanes 1, 3,* and *5*) or *Cla*I (*lanes 2, 4,* and *6*) and transcribed with SP6 RNA polymerase in the presence of capping reagent and [α-^{32}P]ATP. Transcripts containing about 10^6 dpm/μg were then injected into *Xenopus* oocytes (five oocytes for each experiment; Drummonod *et al.*, 1985), and RNA was extracted after 1 h (*lanes 1* and *2*), 3 h (*lanes 3* and *4*), or after 17 h (*lanes 5* and *6*) and analyzed as described (Drummond *et al.*, 1985). *Lanes 8* and *9* show microinjected capped and polyadenylated mRNA derived from the TeTx3'389 deletion mutant after 1 h (*lane 8*) and after 3 h, respectively. In addition polyadenylated TeTx5'10 mRNA was injected and analyzed 1 h (*lane 10*) and 3 h (*lane 11*) after injection.

certain lymphokines, cytokines, and protooncogenes (Shaw and Kamen, 1986).

To see whether polyadenylation stabilized the mRNA encoding the L chain of TeTx, we linearized pSA14 either with *Bam*HI or with *Hin*dIII, *i.e.* immediately upstream or downstream from the dA-dT segment, respectively (Fig. 2*A*). Transcripts labeled with [α-^{32}P]adenosine were separated from nonincorporated nucleotides by passage over a Sephadex G-50 spun column. They were then precipitated, dissolved in 10 mM TE buffer, pH 7.4, and microinjected into *Xenopus laevis* oocytes. This system has been used previously by Drummond *et al.* (1985) to assay the stability of mRNAs exhibiting a balanced G+C content. Total RNA was reisolated at 1, 3, and 17 h after injection and analyzed by electrophoresis on a 2% formaldehyde gel and subsequent autoradiography (Fig. 4, *lanes 1–6*). Polyadenylation clearly increased the stability of the microinjected RNA. This finding is in keeping with our observation that we were unable to detect any depression of postsynaptic responses in the *Aplysia* system (see below) when mRNA was injected which lacked the poly(A) tail. Fig. 4 further demonstrates that mRNA encoding the deletion mutants TeTx3'389 (*lanes 8* and *9*) or TeTx5'10 (*lanes 10* and *11*) were not degraded within 3 h. None of the injected oocytes exhibited detectable morphologic alterations at 17 h after injection that could be assigned to toxic effects of newly synthesized gene products.

Characterization of the Individual Deletion Mutants by Combined in Vitro Transcription/Translation—To verify that the mRNAs obtained by *in vitro* transcription of the individual 5'- and 3'-deletion mutants yielded polypeptides of the expected size, we performed translations in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analyzed the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results obtained for the COOH-terminal L chain deletion mutants of Tetx and BoNT/A are summarized in Fig. 5. Unlabeled Tetx and BoNT/A were applied as controls. TeTx was prepared from single-chain TeTx by treatment with 1 μg/ml *Staphylococcus aureus* protease V8 as described by Weller *et al.* (1988). As shown in Fig. 5*A* the major translation products obtained from wild type TeTx L chain mRNA (*lane 3*) had an electrophoretic mobility like authentic L chain of TeTx. The lower molecular weight material observed in translations of 3'-truncated mRNAs of TeTx and BoNT/A could either result from ribosomal slippage and internal initiation of translation or specific proteolytic degradation within the amino-terminal domain of the L chains. In principal, similar results were obtained with mRNA encoding various 5'-deletion mutants. Again, lower molecular weight species were observed (data not shown). In each case, however, the largest species was the most abundant product the molecular weight of which corresponded to that listed in Table II.

Inhibition of Neurotransmitter Release from Presynaptic Neurons of A. californica upon Injections of L Chain-specific mRNAs—Central synapses of *A. californica* have been used extensively to study the action of clostridial neurotoxins (Mochida *et al.*, 1989; Poulain *et al.*, 1988, 1989a, 1989b). These studies have indicated that TeTx or BoNT/A, B, or E at nanomolar concentrations, when injected into presynaptic neurons, induced a depression of postsynaptic responses caused by inhibition of ACh release from the presynaptic neurons, beginning with a short delay of a few min. As shown in Fig. 6*A*, injection of mRNA derived from pSA14 (encoding the entire L chain of TeTx) also depressed ACh release. however, only after a delay of about 50 min. This delay has been ascribed to the time required for translation of the mRNA, diffusion and accumulation of the translation product within the anticipated site of action in the terminal (Mochida *et al.*, 1990). Stimulation of the *noninjected* presynaptic neuron afferent to the same postsynaptic neuron continued to cause about the same postsynaptic response amplitudes after 200 min (Fig. 6*A*, *open circles*) or even after overnight incubation (not shown) excluding the possibility that the postsyn-

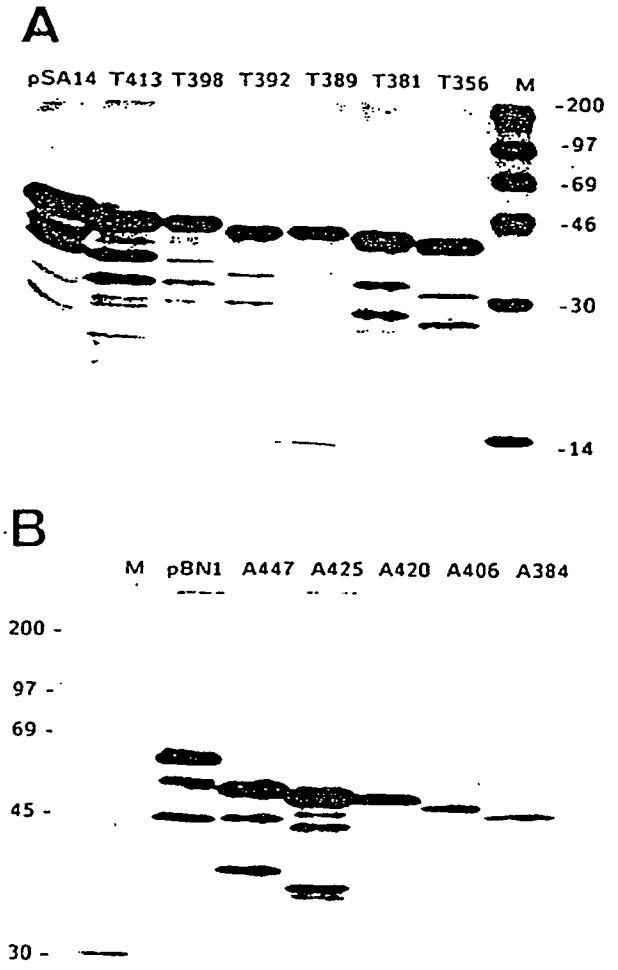


FIG. 5. *In vitro* translation of various mRNAs encoding the carboxyl-terminal deletion mutants of the L chains of TeTx (*A*) and BoNT/A (*B*). *Numbers* indicate the carboxyl-terminal amino acid. *M*, molecular weight markers. The translation reactions and analyses of the translation products on 12 sodium dodecyl sulfate-polyacrylamide gels were performed as described (Mayer *et al.*, 1988).

aptic cell was deteriorated during the experiment. In principle, similar depression curves were obtained with several carboxyl-terminal deletion mutants including TeTx3′392 (Table II; Fig. 6*B*, *filled circles*). This mutant lacked 65 carboxyl-terminal residues of the TeTx L chain including the cysteine residue that had been shown to be involved in the linkage to the H chain (Krieglstein *et al.*, 1990). Apparently, toxicity of this derivative was only slightly reduced, as judged from the time required to reach 50% blockade of neurotransmisssion. However, the additional deletion of Thr^{392}, Asp^{391}, and Asp^{390} in TeTx3′389 (Fig. 6*B*, *open circles*) or of further residues, as is the case in the derivatives TeTx3′381 and TeTx3′356, completely abolished toxicity (Table II).

A similar set of experiments was performed with various carboxyl-terminal deletion mutants of the L chain of BoNT/A (Table II). In accordance with previous data (Mochida *et al.*, 1990), postsynaptic responses remained unchanged after the injection of BoNT/A L chain-specific mRNA. Only when the H chain (50 nM) was added to the bath, an immediate onset of depression was observed (Fig. 7*A*). These findings support previous conclusions that in *Aplysia* both the L and the H chain of BoNT/A are required to generate toxicity

TABLE II

Characterization of mutants of the L chains of TeTx and BoNT/A

Derivative[a]	Terminal sequence[b]	MW[c]	Toxicity[d]
TeTx LC	$MP^{2}ITINN..$	52,412	+
TeTx5′ 8	$MR^{9}YSDP...$	51,613	+
TeTx5′ 10	$MS^{11}DPVN..$	51,293	−
TeTx5′ 12	$MP^{13}VNND..$	50,994	−
TeTx5′ 19	$MI^{20}MMEP..$	50,337	−
TeTx3′ 413	$..EYKG^{413}P$	47,597	+
TeTx3′ 398	$..NDTE^{398}AA$	45,945	+
TeTx3′ 392	$..LDDT^{392}LN$	45,294	+
TeTx3′ 389	$..PNLL^{389}I$	44,849	−
TeTx3′ 381	$..MNHD^{381}A$	43,932	−
TeTx3′ 356	$..SIMY^{356}RLIN$	41,399	−
BoNT/A LC⁻	$..EENI^{454}IDEFELGTTLIN$	57,824	+
BoNT/A5′ 8	$MN^{9}YKDP...$	50,430	+
BoNT/A5′ 10	$MK^{11}DPVN...$	50,153	−
BoNT/A5′ 12	$MP^{13}VNGV...$	49,910	−
BoNT/A5′ 19	$MA^{20}YIKI...$	49,215	−
BoNT/A5′ 45	$MI^{45}PER..$	46,337	−
BoNT/A3′ 188	$..YIRF^{188}RLIN$	21,859	−
BoNT/A3′ 201	$..TNPL^{201}FA$	23,707	−
BoNT/A3′ 254	$..YEMS^{254}PLN$	28,938	−
BoNT/A3′ 315	$..LQYM^{315}A$	35,453	−
BoNT/A3′ 384	$..KVNY^{384}PLN$	44,247	−
BoNT/A3′ 406	$..GQNT^{406}RLIN$	46,887	+
BoNT/A3′ 420	$..KNFT^{420}RLIN$	48,583	+
BoNT/A3′ 425	$..LFEF^{425}RLIN$	49,177	+
BoNT/A3′ 447	$..KGYN^{447}PLN$	51,487	+

[a] 3′-deletions were introduced by exonuclease III digestion; 5′-deletions were generated by the polymerase chain reaction. mRNA was injected into the identified presynaptic neuron.

[b] All clones were characterized by direct sequencing. Numbers specify the last or the first authentic amino acid residue of the individual L chains. Dots indicate that the protein continues with the authenic amino-terminal or carboxyl-terminal sequences.

[c] Theoretical molecular weights including the start methionine as deduced from the DNA sequence.

[d] Depression (+) or failure of depression (−) of evoked postsynaptic responses were determined in at least three separate experiments in which mRNA was microinjected into the identified presynaptic cholinergic neuron of the buccal ganglia of *A. californica*.

(Poulain *et al.*, 1988). However, it must be stressed that neither bath-applied nor internally applied H chain caused any change in the postsynaptic response even when applied at concentrations of up to 125 nM (Maisey *et al.*, 1988; Poulain *et al.*, 1990).

Theoretically, the pBN1-derived mRNA should yield a translation product that contains the entire L chain, 46 amino acid residues form the H_N region and 12 foreign residues specified by the vector (Table II). The conclusion that such additional carboxyl-terminal sequences do not interfere with toxicity, however, is not justified for two reasons. First, we cannot exclude proteolytic processing of the primary translation product at the nicking site. Second, because of the extreme neurotoxicity, undetectable amounts of truncated peptides arising from premature termination of translation could account for the toxic effects.

A deletion of the 63 carboxyl-terminal amino acids from the L chain of BoNT/A, as present in BoNT/A3′384, abolishes toxicity (Fig. 7*C*), whereas a derivative that is 22 residues longer retains full activity (Fig. 7*B*). From the deletion mutants summarized in Table II we can conclude that Cys^{439} of TeTx and Cys^{430} of BoNT/A are not required to evoke neurotoxicity. This finding argues against the possibility that these cysteines have to become posttranslationally modified, for instance by polyisoprenylation, as observed for a variety of biologically active molecules such as the *ras* and *ras*-related small GTP-binding proteins (Hancock *et al.*, 1989) or the

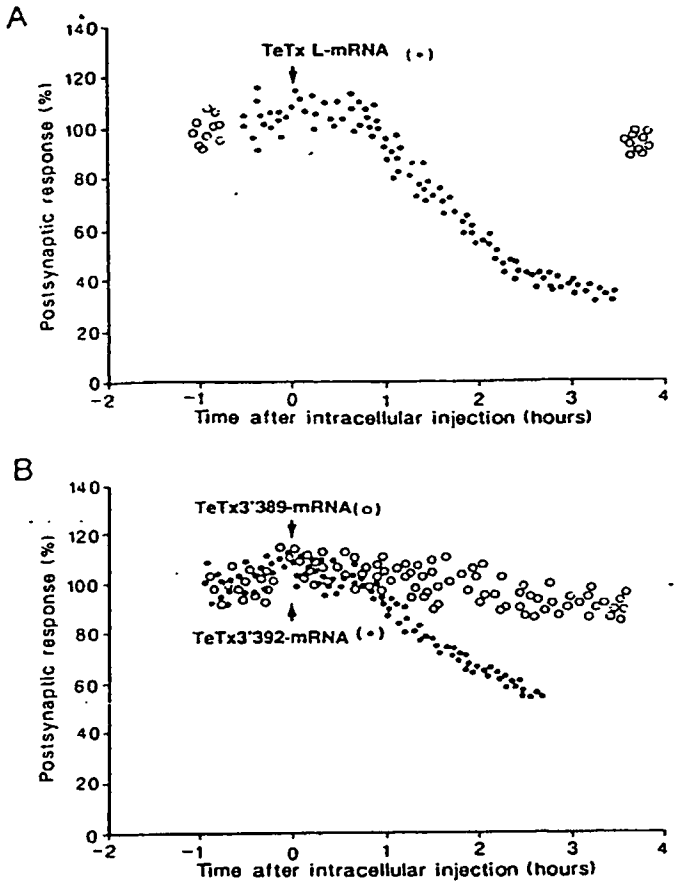


FIG. 6. Depression of ACh release following injection of TeTx L chain-specific mRNA into the presynaptic neuron in the buccal ganglion of *Aplysia*. Amplitudes of postsynaptic responses (expressed in percent of control responses before injection) elicited by evoked presynaptic action potentials in two presynaptic neurons were plotted against the time. *A*: *filled circles*, after control recordings, mRNA (0.5 $\mu g/\mu l$) derived from pSA14 was pressure injected into one of the presynaptic neurons. Depression of postsynaptic responses occurring after about 50 min indicates inhibition of transmitter release. *Open circles*, control responses, recorded from the same postsynaptic neuron, were elicited by stimulation of the noninjected second presynaptic neuron. *B*: mRNA derived from TeTx3'391 (*filled circles*) or TeTx3'389 (*open circles*) were injected into different presynaptic neurons. Note that although the translation product of the former mRNA was toxic, that from the latter mRNA was inactive. All mutants were tested in at least three different ganglia.

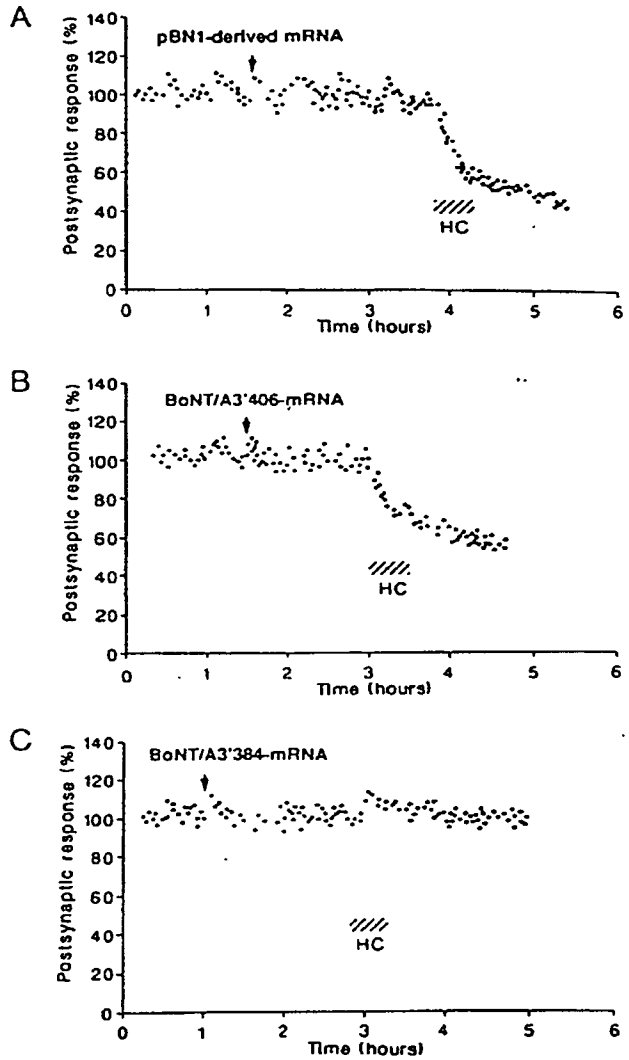


FIG. 7. Inhibition of transmitter release upon injection of mRNA encoding various L chain-specific deletion mutants of BoNT/A. *A*, injection of mRNA encoding the entire L chain of BoNT/A plus 58 additional carboxyl-terminal amino acid residues ($n = 3$). Depression of transmitter release occurred only after addition of BoNT/A H chain (50 nM, *HC, hatched area*) to the bath. *B*, mRNA derived from BoNT/A3'406 ($n = 4$) was injected. Again, ACh release decreased after addition of the H chain. *C*, BoNT/A3'384 mRNA ($n = 5$) was injected. No depression was observed after posttranslational bath application of the H chain of BoNT/A. Similar results were obtained when the preparation was preloaded with H chain prior to injection of mRNA. These data demonstrate that the corresponding L chain derivative and the H chain on their own are nontoxic.

nuclear lamins (Krohne *et al.*, 1989). Furthermore, these data exclude any role for the conserved cysteine residues either in the toxification process itself, in a potential attachment to intracellular membranes, or in the interaction of the BoNT/A L chain with the synergistically acting H chain.

We then analyzed various amino-terminal deletion mutants, as generated by PCR of the corresponding genes. The results are summarized in Table II and in Fig. 8. Whereas a deletion of 8 residues is tolerated in both the L chains of TeTx and BoNT/A without affecting their toxicity, a deletion of the conserved tyrosine residue in position 10, or of additional amino acid residues (Table II), completely abolished toxicity (Fig. 8). At present it is unclear whether Tyr^{10} plays a role in merely stabilizing the tertiary structure of the individual L chains. Alternatively, this residue could be involved in binding to the putative subcellular target molecule or in the interaction with an, as yet undefined, cellular cofactor.

In general, the above conclusions are based on the assumption that the various translated protein products are equally resistant against proteolytic degradation in the microinjected *Aplysia* neuron. Since we were as yet unable to detect the corresponding polypeptides by Western blotting, processing of the gene products cannot be excluded.

The H_C Domain of the H chain of BoNT/A Synergistically Activates the L Chain—The well documented synergistic function of the botulinal H chains in the *Aplysia* system (Maisey *et al.*, 1988; Poulain *et al.*, 1988) is not understood, even more so since several reports have demonstrated that the same preparations of botulinal L chains alone are blocking transmitter release when introduced into the cytosol of bovine chromaffin cells (Bittner *et al.*, 1989; Stecher *et al.*, 1989b), PC12 cells (McInnes and Dolly, 1990), or into mammalian

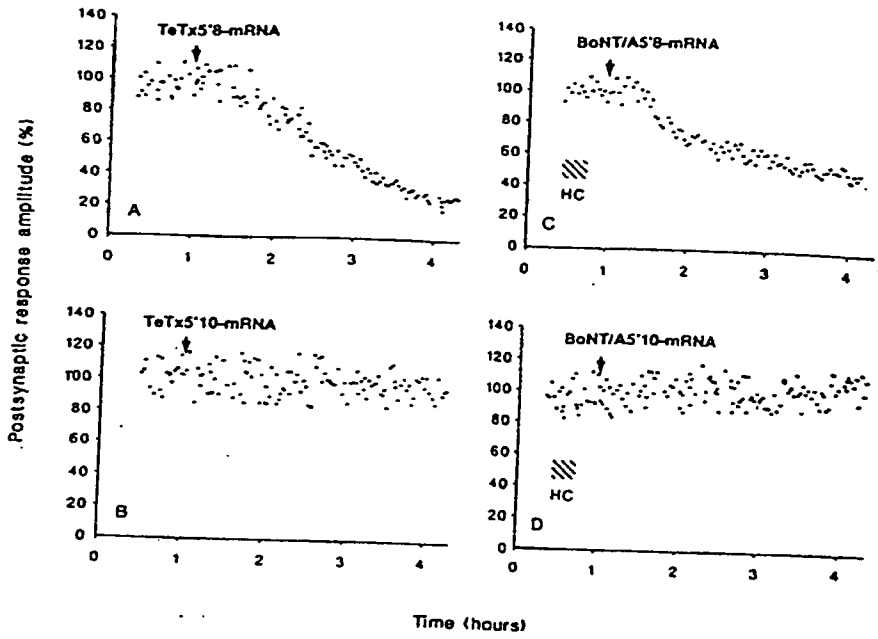


FIG. 8. Analyses of amino-terminal deletion mutants of the L chain of TeTx and BoNT/A. *A* and *B*, mRNA (0.5 μg/μl) encoding either TeTx5'8 (n = 3) or TeTx5'10 (n = 4) was injected as above, and evoked postsynaptic resonse amplitudes were recorded. A TeTx L chain derivative lacking 8 amino-terminal residues is neurotoxic, albeit at reduced potency, as judged by the time required to reach 50% depression (compare Fig. 6*A*). In contrast, a derivative lacking 10 amino-terminal residues is nontoxic. *C* and *D*, analyses of the corresponding amino-terminal L chain deletion mutants of BoNT/A, however in the presence of bath-applied H chain added prior to injection of the mRNA (*HC, hatched area*).

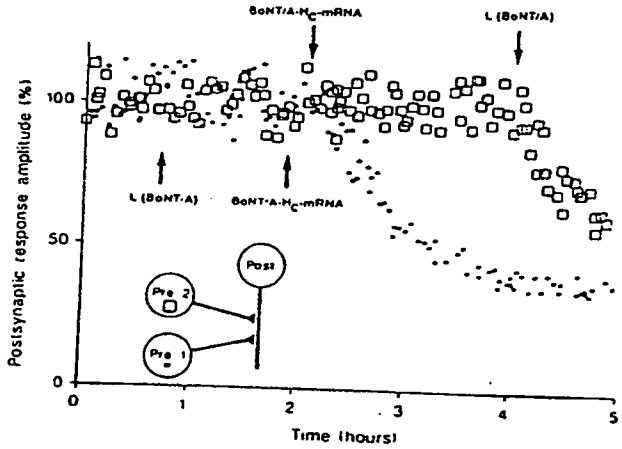


FIG. 9. The H_C portion of the H chain of BoNT/A is required the cytosol of the presynaptic neuron to allow BoNT/A L ain-specific blockade of neurotransmitter release. In this periment the two identified presynaptic cells afferent to the same stsynaptic cell were microinjected with H_C-specific mRNA either er injection of the purified BoNT/A L chain peptide (pre 1; *filled ıares*) or prior to injection of the toxifying L chain (pre 2; *open ıares*). For further details see "Results and Discussion."

rve terminals (de Paiva and Dolly, 1990). The *Aplysia*- tricted helper function mediated by the botulinal H chain s been assigned to its H_C domain based on the following es of indirect evidence: L-H_N of BoNT/A, a proteolytic avage product isolated from the di-chain toxin (Fig. 1), was nd to be inactive in *Aplysia* neurons, no matter whether lied extra- or intracellularly (Poulain *et al.*, 1989a). Neuoxicity was restored, however, by bath application or miinjection of the entire H chain from BoNT/A, but not n TeTx (Poulain *et al.*, 1990). Unfortunately, the contriion of H_C to the toxification process could not be assessed ctly because of practical problems associated with the ation of this peptide fragment in its native form (Shone ., 1985). To overcome this problem, we generated a DNA ment by PCR that encoded the entire H_C region (residues ^{872} to Leu^{1296} of the BoNT/A sequence published by Binz *et al.*, 1990a). The PCR product was resequenced and analyzed by combined *in vitro* transcription/translation. A single molecular species (M_r 45,000) was obtained (data not shown). We then applied the corresponding mRNA in microinjection experiments, as shown in Fig. 9. One of the two identified presynaptic cholinergic neurons (pre 1) was first injected with the purified L chain of BoNT/A (20 nM calculated intracellular concentration). As expected, no inhibition of neurotransmitter release was observed within 90 min after injection (*small filled squares*). At this time point the H_C-specific mRNA was injected into the same neuron (pre 1). After a delay of about 35 min an onset of depression was observed indicating that, indeed, the H_C fragment mimicked the effects previously provided by the entire H chain. As a control, we first injected the second presynaptic neuron (pre 2) with H_C-specific mRNA. Evoked transmitter release remained unaltered for 2 h indicating that the H_C peptide by itself generated no neurotoxic effects (Fig. 8, large open squares). The subsequent injection of purified BoNT/A L chain into the same neuron caused an immediate onset of depression of the postsynaptic response.

Together, these data clearly demonstrate that in the *Aplysia* neurons the H_C domains of BoNT/A has to be exposed in the cytosol in order to allow the BoNT/A L chain to block neurotransmitter release. It has frequently been argued that the *Aplysia* synapses were too distant from vertebrate synapses on the evolutionary scale and that, therefore, the former synapses lacked a property or function being provided by the vertebrate nerve terminals. Indeed, the synergistic role of the H_C fragment raises a number of questions.

1. How does the H_C domain reach the cytosol? Does such translocation of H_C also occur in the neuron of higher organisms? At present, we can only speculate about such mechanism. If we assume that internalization of BoNT/A into *Aplysia* neurones takes place by receptor-mediated endocytosis, as documented for the uptake into motor nerves (Black and Dolly, 1986), the intact toxin molecule should then become delivered via an endosomal compartment into smooth-walled vesicles characterized by an internal low pH. Under these conditions the H chain of clostridial neurotoxins were reported to form pores (Boquet and Duflot, 1982; Boquet *et*

al., 1984; Shone *et al.*, 1987; Hoch *et al.*, 1985; Donovan and Middlebrook, 1986). Exposure of the H_C domain of BoNT/A to the cytosol could occur concomitantly with the postulated translocation of the L chain, perhaps in close association with it. We could envisage a flipping mechanism by which the intravesicularly located H_C domain traverses, together with the L chain, the pore generated by the H_N portion of the H chain. Such alteration of the membrane topology of H_C could be verified by immunocytochemistry using H_C-specific monoclonal antibodies.

2. Is there any proteolytic processing of the H chain after internalization of the intact toxin? In that case a protease that would liberate the H_C portion could be present in mammalian neurons but could be absent in *Aplysia* (Poulain *et al.*, 1990). This would, however, leave us with the question of how H_C then penetrates the vesicular membrane.

3. Is the synergistic role of the H_C domain based on a direct physical interaction between L and H_C, perhaps by stabilizing the conformation of the L chain? Alternatively, H_C could mimick an unknown function in the cytosol that is provided by vertebrate cells.

4. Which portion of the BoNT/A L chain requires the helper function of H_C, and why is the L chain of TeTx alone sufficient to block neurotransmitter release? At least this question could be approached directly by the application of genetically engineered chimeric L chains consisting of defined portions of TeTx and BoNT/A.

Answers to the above questions will provide additional insights into the mechanisms by which the clostridial neurotoxins operate. At any rate, the *Aplysia* system combined with mRNA microinjection techniques appears to open a safe way to study the function of individual subdomains of clostridial neurotoxins following genetic engineering of their genes.

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